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NUCLEAR TRANSFER WITH SELECTED DONOR CELLS

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~~The present invention concerns a novel method of nuclear transfer, specifically, although by no means exclusively for use in cloning technologies for the production of mammalian embryos, fetuses and offspring, including genetically engineered or transgenic mammalian embryos, fetuses and offspring.~~

BACKGROUND

The stage of cell cycle of the donor nucleus and the recipient cytoplast at the time of embryo reconstruction are important factors determining successful development following nuclear transfer. Specific combinations between the two "cells" are required to ensure a diploid set of chromatin following the first embryonic cell cycle and to maximise the opportunities for nuclear reprogramming and subsequent development. When an interphase donor nucleus is fused with an enucleated metaphase-arrested oocyte (termed the cytoplast or recipient cell), there is immediate nuclear envelope breakdown (NEBD) and the donor chromatin undergoes premature chromosome condensation (PCC)(Barnes *et al.*, 1993). These effects are induced by a cytoplasmic activity termed maturation promoting factor (MPF, alternatively called meiosis- or mitosis-promoting factor; see review by Campbell *et al.*, 1996a). MPF activity during oocyte maturation is maximal at metaphase stages and declines rapidly upon either fertilisation or artificial activation. Thus, two types of cytoplast may be used for reconstruction; those either high or low in MPF using either non-activated or activated metaphase II (MII) cytoplasts, respectively.

Early studies which helped gain an understanding of the importance of cell cycle co-ordination in mammalian nuclear transfer for maintaining chromosome integrity and hence developmental potential, were performed with undifferentiated blastomeres (*ie* non-specialised embryonic cells) from pre-implantation stage embryos in species such as rabbit, sheep and cattle. These studies revealed that the stage of the cell cycle of the donor nucleus and the length of exposure to MPF in the cytoplast have marked effects

on the degree of PCC observed. The chromatin of S-phase nuclei exposed to MPF has a typically pulverised appearance and chromosomal studies reveal a high incidence of abnormalities (Collas *et al.*, 1992b). In contrast, with nuclei at G1 or G2 the chromatin condenses to form elongated chromosomes with either single- or double-stranded chromatids, respectively (Collas *et al.*, 1992b). Following a suitable stimulus to release the reconstructed embryo from metaphase-arrest and to activate development, the nuclear envelope reforms around the donor chromatin which then undergoes DNA synthesis regardless of its previous cell cycle stage. Thus, donor nuclei- in G1 initiate DNA synthesis which is compatible with normal development, while nuclei in G2 or S-phases either completely or partially re-replicate already replicated DNA so that by the end of the first embryonic cell cycle the DNA content in the two daughter cells will be incorrect leading to abnormal early embryonic development.

In contrast, these early studies demonstrated that when blastomere nuclei are transferred to cytoplasts after the disappearance of MPF, following a sufficient interval after cytoplast activation, NEBD does not occur (so therefore neither does PCC) and it is the donor nucleus which controls DNA replication in accordance with its stage in the cell cycle at the time of transfer. Thus, nuclei in G1 or S-phases initiate or continue replication, respectively, while those in G2 are not induced to enter another round of DNA synthesis. Such pre-activated cytoplasts have been termed "universal recipients" (Campbell *et al.*, 1994) and are capable of co-ordinating the development of donor cells at any stage of the cell cycle. This has been especially important for cloning pre-implantation embryos where most undifferentiated blastomere nuclei are in S-phase at any one time (80-90%; Barnes *et al.*, 1993; Campbell *et al.*, 1994) and are therefore most compatible with transfer to cytoplasts low in MPF.

Following nuclear transfer, normal development will depend upon factors present within the oocyte cytoplasm (or additional factors introduced exogenously) being able to remodel chromatin structure and to appropriately reprogramme the pattern of gene expression of the donor nucleus. The mature cytoplast contains the RNA transcripts and proteins to direct development of the normally fertilised cleavage-stage embryo up

to the normal time of genome activation, when embryonic nuclei begin the synthesis of their own RNA to direct embryogenesis. Donor nuclei obtained from embryos or cell types which have already passed this point must therefore cease their RNA synthesis after reconstruction and remain inactive until the newly reprogrammed maternal-embryonic genome transition occurs. Following transfer, the donor nucleus is forced to reprogram to the zygotic state, and subsequently activate the appropriate genes at the correct levels, in the proper temporal and spatial manner for normal embryo development to occur. The mechanisms that achieve such nuclear reprogramming are currently not well understood.

Reprogramming and cell cycle co-ordination have become important topics given the recent interest in performing nuclear transfer with cells that can be maintained in culture. These cultured cells are more differentiated (*ie* possess a more specialised cellular function) that those used in the earlier studies with embryonic blastomere cloning. These cells can be isolated from either embryos, fetuses or adult animals. Because there is access to larger numbers of cells, efficient nuclear transfer techniques with these differentiated cell cultures would enable in livestock species large scale multiplication of desirable genotypes and would facilitate the production of transgenic animals following genetic manipulation of the cultured cells

Early studies with actively growing, unsynchronised cultures of ovine embryonic cells (cell cycle stage unknown) fused to pre-activated cytoplasts did in fact produce lambs at term from early (Campbell *et al.*, 1995) but not later passage cells (Campbell *et al.*, 1996b). Subsequent studies, where the cells were deprived of serum for 5 days and reported to be quiescent (*ie* whereby the cells supposedly exited the normal cell division cycle and entered a so-called "G0" state), yielded viable lambs following fusion with cytoplasts either before, after or simultaneous with activation at similar overall efficiencies (Campbell *et al.*, 1996b). These authors (Campbell *et al.*, 1996b; Schnieke *et al.*, 1997; Wilmut *et al.*, 1997; Patent WO 97/07669) suggest the importance of using cells that have exited the normal cell division cycle and are synchronised in a quiescent or G0 state to facilitate nuclear reprogramming and enable

the production of cloned animals from differentiated cells. The preferred method of synchronising cells in quiescence (based on absence of proliferating cell nuclear antigen (PCNA) indicating no cells in S-phase) by the above authors has been by serum starvation for a suitable period of time. Recently, however, it has been questioned as to what proportion of serum starved cells are actually in G0. Using dual parameter flow cytometry to simultaneously measure both cellular DNA and protein content (to distinguish between G0 and G1 cells in the diploid population, with quiescent cells having less RNA and protein) Boquest *et al.* (1999) investigated the cell cycle characteristics of cultured fetal pig fibroblasts. They demonstrated that despite serum starvation for 5 days, less than 50% of cells were actually in G0 by their definition. By selecting "small" cells in the population, the proportion in G0 increased to 72% in the serum starved cultures (Boquest *et al.*, 1999).

As an alternative to the use of quiescent cells, Cibelli and colleagues (1998; and patent specification WO 99/01163) reported the use of non-serum starved, randomly growing cultures of bovine cells fused to MII cytoplasts which were subsequently activated using ionomycin and 6-dimethylaminopurine (6-DMAP). However, it is not clear from these disclosures what the donor cell cycle stage was at the time of nuclear transfer in those resulting embryos that, ultimately yielded the cloned calves by these methods.

Similarly, there are other reports whereby cloned calves (Vignon *et al.*, 1998, 1999; Zakhartchenko *et al.*, 1999) and mice (Wakayama and Yangimachi, 1999) have been produced from non-serum deprived, randomly growing cell cultures. However, again, the stage of the donor cell cycle at the time of nuclear transfer which resulted in the cloned offspring remains uncertain.

Thus, none of these aforementioned studies have demonstrated exactly what stage or stages of the cell cycle have resulted in that low proportion of reconstructed embryos which ultimately develop into viable offspring.

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The above discussion highlights that to date there has been generally poor characterisation as to the stage of the cell cycle cultured donor cells are in at the time of nuclear transfer, which makes the state of the art uncertain and not easily replicated.

It would therefore be desirable to have a method of nuclear transfer which ensured that the stage of cell cycle of the donor nuclei were accurately known.

It is an object of the present invention to go some way towards achieving this desideratum or at least to provide the public with a useful choice.

SUMMARY OF THE INVENTION

According to a first aspect, the present invention provides a method of nuclear transfer, comprising selecting and segregating G1 cells from a proliferating or non-proliferating population of diploid donor cells and transferring a nucleus from such a segregated G1 cell into an enucleated recipient cell, wherein donor cells are selected and segregated by physical picking based on individual cell identification to produce a pure G1 cell population. The donor cell population may be at one or more known or unknown stages of the cell cycle, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised by ≥5μM nocodazole or 5 μg/ml colcemid.

Such a method provides certainty as to the stage of the cell cycle of the donor nuclei at the time of embryo reconstruction and is therefore advantageous over the prior art.

The invention contemplates the use of cell cycle inhibitors, (other than ≥5μM nocodazole or ≥5μM colcemid) to block randomly growing cells at specific stages of the cell cycle to produce a non-proliferating synchronised cell population. Preferably cell growth is blocked at mitosis and, following release from the inhibitor, cells progressing into G1 phase are used for nuclear transfer.

Thus, in a second aspect, the invention provides a method of nuclear transfer which comprises transferring a diploid nucleus from a cell segregated from a non-proliferating cell population which has been synchronised in the G1 stage of the cell cycle, into an enucleated recipient cell, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronished at G1-phase by ≥5μM nocodazole or 5 μg/ml colcemid.

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Preferably said G1 cells are individually segregated from a randomly proliferating population or from a non-proliferating synchronised cell population at an early G1 phase.

Alternatively, the non-proliferation cell population may comprise senescent cells.

The segregated G1 donor cell is isolated from an animal *in vivo* or, more preferably, from a cell culture *in vitro*. Suitable cells may be derived from either embryos, fetuses, juvenile animals, through to fully mature adults. Practically any diploid karyotypically normal cell that is capable of cell proliferation or a senescent cell could be used in the current invention. Cells could be of an undifferentiated cellular state or at any varying degree of cellular differentiation so long as they can be stimulated to enter the cell cycle and proliferate. Cells which are quiescent could be stimulated to enter the cell cycle with the appropriate culture conditions (such as by the addition of serum or specific growth factors) and used for nuclear transfer in an early G1 state following mitosis. Some cell types may well prove to be more efficient than others, however, both adult and fetal fibroblasts and adult follicular cells have been found to be satisfactory. By way of demonstration of the invention, results are presented below in the bovine species (in examples 1-7) using two follicular cell lines, four skin fibroblast cell lines and two genetically modified fetal fibroblast cell lines.

Preferably the recipient cell comprises an enucleated oocyte obtained from a species corresponding in origin to the donor nuclei. The enucleated oocyte may have either high or low MPF activity at the time of embryo reconstruction as either state is compatible with a G1 donor nucleus with a 2C amount of DNA.

Alternatively, the recipient cell may comprise an enucleated stem cell or a clump of enucleated stem cells fused together. Preferably these stem cells are embryonic stem cells. Such embryonic stem cells that are used as the recipient cells in nuclear transfer may be themselves isolated from a growing embryo or from already established stem cell lines in culture. In this case, nuclear transfer using donor nuclei from G1 cells

selected by the method of the invention may be carried out for the purposes of "therapeutic cloning".

According to a third aspect, the present invention provides a method of producing cloned animal embryos by transferring a donor diploid nucleus from a cell selected and segregated in G1 phase according to the invention, preferably early G1 phase, into an enucleated recipient cell, with the proviso that said diploid donor cells are not selected from blastomeres which have been synchronised at G1-phase by $\geq 5\mu M$ nocodazole or 5 μg/ml colcemid.

The methods of the present invention may be used to produce any animal embryo species of interest including birds, amphibia, fish and mammals. Preferably the animal embryo of interest is a mammal, including, but not limited to, primates including humans, rodents, rabbits, cats, dogs, horses, pigs and most preferably, ungulates such as cattle, sheep, deer and goats.

Preferably the cloned animal embryos have desirable genetic traits using genetically modified nuclei by methods known in the art.

According to a fourth aspect, the present invention provides a reconstituted animal embryo prepared by the methods of the invention including a reconstituted transgenic animal embryo. The embryos so formed may then be either re-cloned to further increase embryo numbers or undergo serial nuclear transfer to further aid nuclear reprogramming and/or developmental potential.

According to a fifth aspect, the present invention provides a method of cloning a non-human animal comprising (1) producing a cloned animal embryo according to the method of the invention described above; (2) allowing an animal to develop to term from the embryo by known methods; and (3) optionally breeding from the animal so formed either by conventional methods or by cloning according to the methods of the present invention.

The methods of the present invention may be used to produce a non-human animal species of interest including birds, amphibia, fish and mammals. Preferably said non-

human animal is selected from the group comprising non-human primates, rodents, rabbits, cats, dogs, horses, pigs and most preferably ungulates such as cattle, sheep, deer and goats.

According to a sixth aspect, the present invention provides a cloned non-human animal prepared by the methods of the invention, described above, as well as to the offspring and descendants of such cloned non-human animals.

The methods of the present invention may be used to produce non-human animals, preferably mammals, having desirable genetic traits using genetically altered donor nuclei by methods well known in the art. Transgenic animals produced by such methods also form part of the present invention.

The present invention can also be used to produce embryonic cell lines, embryonic stem cells, fetuses or offspring which can be used, for example, in cell, tissue and organ transplantation.

Accordingly, in a further embodiment, the present invention provides a method of producing a cell line comprising the steps a) selecting and segregating G1 cells from a proliferating population of diploid donor cells of unknown cell cycle or from a synchronised population of diploid G1 cells and transferring a nucleus from such a segregated cell into an enucleated recipient cell; b) optionally growing to embryo stage; c) recovering cells; and d) establishing an immortalised cell line *in vitro* by methods known in the art.

In a further embodiment there is provided a method of producing animal stem cells comprising the steps of a) selecting and segregating G1 cells from a growing population of animal diploid donor cells at various unknown stages of the cell cycle, or from synchronised cultures of diploid G1 cells, and transferring the nucleus from such segregated cells into an enucleated animal recipient cell; b) optionally growing to embryo stage; c) recovering stem cells.

Preferably, the stem cells are embryonic stem cells.

Preferably, the animal donor cell used in the above methods is of human origin. Most preferably, both the animal donor and recipient cells used in the above methods are of human origin.

Most preferably, the donor cells are adult or fetal cells selected from any karyotypically normal cell type and the recipient cells are selected from any cell type capable of reprogramming gene expression including enucleated oocytes or embryonic stem cells.

The embryonic stem cells produced by the method of the invention are pluripotent and may be induced to differentiate in culture to form purified populations of specialised types of human cells including nerve cells such as neurons, astrocytes, oligodendrocytes; liver cells; muscle cells such as myocytes; heart cells such as cardiomyocytes, haematopoietic cells, pancreatic cells and any other cell type of interest by methods known in the art.

Such specialised human cells and tissues may then be used for transplantation for treatment of specific diseases or injuries where the damaged cells are unable to replace themselves or replace themselves effectively. Where the human donor cell was derived from a patient in need of such a transplant, such a transplanted tissue would not be rejected by the patient as the tissue would be genetically identical to the patient.

Alternatively, such differentiated cells and tissues could be used to treat diseases or injuries, for example, various neurological disorders (*eg* Parkinson's disease), diabetes, heart disease, muscular dystrophy, various hereditary diseases, specific cancers (*eg* leukemia), spinal cord injury, burns and other afflictions.

These methods are known as "therapeutic cloning" in the art.

The *in vitro* differentiation of human embryonic stem cells to specific cell types may be also beneficial for drug discovery and toxicology studies for human medicine.

Thus, in a further aspect, the present invention provides a method of drug discovery and toxicology testing of drugs using *in vitro* differentiated human embryonic stem cells produced by the methods of the present invention.

According to a still further aspect, the present invention provides a method of xenotransplantation whereby cells, tissues and organs may be isolated from cloned non-human animals and their offspring produced according to the methods of the invention, and used for transplantation in human patients in need of such therapy. Where such cells, tissues or organs comprise a transgene, such cells, tissues or organs may be useful in gene therapy or to moderate the patient's immune response to the xenogenic tissue.

DESCRIPTION OF THE FIGURES

The present invention will now be described by reference to the figures of the accompanying drawings in which:

Figure 1 shows the survival rate throughout the gestation of cloned cattle embryos reconstructed with follicular cells either in G0 or G1 of the donor cell cycle;

Figure 2 shows the survival rate throughout gestation of cloned cattle embryos reconstructed with adult female skin fibroblasts either in G0 or G1 of the donor cell cycle;

Figure 3 shows the survival rate throughout gestation of cloned cattle embryos reconstructed with adult female skin fibroblasts (3XTC cells) either in G0 or G1 of the donor cell cycle;

Figure 4 shows embryonic survival throughout gestation of cloned cattle embryos reconstructed with adult male skin fibroblast cells (LJ801 cells) either in G0 or G1 of the donor cell cycle;

Figure 5 shows embryonic survival throughout gestation of cloned transgenic cattle embryos reconstructed with genetically modified female fetal lung fibroblast cells (casein plus 5110 cells) either in G0 or G1 stages of the donor cell cycle; and

Figure 6 shows embryonic survival throughout gestation of cloned transgenic cattle embryos reconstructed with non-proliferating, senescent female fetal fibroblasts (561 cell line).

DETAILED DESCRIPTION OF THE INVENTION

The present invention is directed to an improvement of the known techniques for the cloning of mammalian embryos by nuclear transfer. Although it is contemplated that the embryo cloning procedure of the present invention may be utilised in a variety of mammals and, indeed other animal species, the procedure will be described with reference to the bovine species. It is an essential feature of this invention that the donor nuclei are diploid and are in G1 phase, more preferably in early G1 phase.

One approach in obtaining G1 cells with certainty from a randomly growing population of cultured cells is to individually pick mitotic cells from the culture surface, and allow them to complete mitosis in medium containing 10% fetal calf serum (FCS). Such donor cells are then fused to recipient cells, (*ie* cytoplasts) within a short period of time following mitosis, such as three hours in common practice and before entry into S-phase, as may be detected by BrdU labeling. In this way they are assured of being in the early phase of G1 and possess a 2C amount of DNA. Thus, the cycling cells are used for nuclear transfer before they have progressed to the G1/S boundary. The selected cells remain in high serum-containing medium throughout the manipulations,

at least until after fusion with the cytoplast has been completed. Thus, the cells are not induced to exit the cell division cycle and do not become quiescent at any point.

Although the present invention contemplates the production of a synchronised population of G1 cells for use in nuclear transfer, one advantage of the methods of the present invention is that they do not necessitate the use of potentially cyto-toxic or perturbing cell synchronisation agents such as nocodazole or colchicine to, for example, pre-synchronise a higher proportion of cells in M-phase before subsequent release from this block and selection of cells in early G1 following cell division. However, in order to select larger numbers of G1 cells or to reversibly arrest cells at specific points during the G1 phase for use in nuclear transfer, it may be advantageous, according to the present invention, to utilise suitable reagents i.e. cell cycle inhibitors at appropriate drug concentrations and incubation times. Such reagents and methods will be known to people skilled in the art. Methods may include a pre-synchronisation treatment to reduce drug exposure times, by for example, temporarily inducing cells to enter G0 by serum deprivation before re-adding serum and allowing the cells to re-enter at the G0/G1 boundary or restriction point and progress through the cell division cycle. Suitable reagents to reversibly arrest cells in various points in G1 (as shown in Gadbois *et al.*, 1992 and references therein) include: (1) staurosporine (a non-specific kinase inhibitor used at extremely low concentrations in the nano-molar range); (2) more specific kinase inhibitors on cell cycle progression, such as for example inhibitors of cAMP-dependent protein kinase and cGMP-dependent protein kinase; (3) lovastatin; (4) isoleucine deficiency; and (5) aphidicolin or hydroxyurea used in a manner to prevent entry into S-phase and block cells at the G1/S boundary.

Since G1 donor nuclei have a 2C amount of DNA (i.e. it is diploid) they may be reconstructed with cytoplasts possessing either a high or low amount of MPF. That is to say, G1 nuclei may be introduced into cytoplasts either before, after or at the same time as activation occurs. However, for improved development of the cloned embryos it is preferable to expose the donor nucleus (introduced either following electrically-induced cell fusion or direct nuclei injection) to factors present within the cytoplasm of

the enucleated oocyte for a suitable period of time in order to facilitate nuclear reprogramming. This has been termed "fusion before activation" or FBA. Previous work has demonstrated the benefits of this approach compared to essentially "simultaneous fusion and activation" or AFS (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999). Furthermore, it is recommended that exposure to the cytoplasm be at least greater than one hour duration and preferably between 3-6 hours in order to improve rates of development to the blastocyst-stage. With this method, however, it is important to prevent by some suitable means the micro-nuclei formation which occurs when fusion precedes activation (Czolowska *et al.*, 1984) in order to maintain the correct ploidy in the resulting embryo.

Below is outlined a method for reconstructing and producing cloned embryos, in the bovine species, derived from both G0 (control) and G1 cultured donor cells. In this particular example described below, bovine follicular cells collected from ovarian follicles were used with results presented in examples 1-3 (the use of fibroblast cell lines is illustrated in examples 4-7). In practice, essentially any cell type possessing a normal diploid karyotype, including embryonic, fetal, juvenile and adult cells, which is either proliferating or can be induced to enter the cell cycle or senescent may prove totipotent using this technology. In addition, any other method known in the art may be used to reconstitute and produce cloned embryos as would be appreciated by a person skilled in the art.

In Vitro Maturation of Oocytes

Slaughterhouse ovaries were collected from female cattle and placed in saline (30° C) and transported within 2 hours to the laboratory. Cumulus-oocyte complexes (COCs) were recovered by aspiration of 3-10 mm follicles using an 18-gauge needle and negative pressure. (Alternatively, immature oocytes could be collected from donor cows via ovum pick-up and subsequently matured *in vitro*). COCs were collected into HEPES-buffered Tissue Culture Medium 199 (H199; Life Technologies, Auckland, New Zealand) supplemented with 50 µg/ml heparin (Sigma, St. Louis, MO) and 0.4%

w/v BSA (Immuno-Chemical Products (ICP), Auckland, New Zealand). Before *in vitro* maturation, only those COCs with a compact, non-atretic cumulus oophorous-corona radiata and a homogenous ooplasm were selected. They were washed twice in H199 medium + 10% FCS (Life Technologies) before being washed once in bicarbonate-buffered Tissue Culture Medium 199 medium + 10% FCS. Ten COCs were transferred in 10 µl of this medium and placed into a 40 µl drop of maturation medium in 5-cm petri dishes (Falcon, Becton Dickinson Labware, Lincoln Park, NJ) overlaid with paraffin oil (Squibb, Princeton, NJ). The maturation medium comprised Tissue Culture Medium 199 supplemented with 10% FCS, 10 µg/ml ovine FSH (Ovagen; ICP), 1 µg/ml ovine LH (ICP), 1 µg/ml oestradiol (Sigma), and 0.1 mM cystamine (Sigma). Microdrop dishes were cultured at 39° C in a humidified 5% CO_2 in air atmosphere for 18-20 hours. After maturation, the cumulus-corona was totally removed by vortexing COCs in 0.1% hyaluronidase (from bovine testis; Sigma) in HEPES-buffered Synthetic Oviduct Fluid (HSOF; Thompson *et al.*, 1990) for 3 minutes, followed by three washes in HSOF + 10% FCS.

Nuclear Transfer with Cultured Cells

a) **Media** Matured oocytes, cytoplasts and reconstructed embryos were either held or manipulated in H199-based media for the period following maturation and until fusion was assessed 15-30 minutes after the electrical pulses. Embryos that were reconstructed by fusing donor cells and MII cytoplasts 3-6 hours before activation (FBA treatment) were cultured in AgResearch Synthetic Oviduct Fluid medium (AgR SOF; which is a modified formulation to that described in Gardner *et al.*, 1994, and is commercially available from AgResearch, Hamilton, New Zealand) minus calcium + 10% FCS until just before activation some 3-6 hours later. Following this point, calcium was present in all media formulations used.

b) **Enucleation** Oocytes matured for approximately 18-20 hours were enucleated with a 15-20 µm (external diameter) glass pipette, by aspirating the first polar body and the MII plate in a small volume of surrounding cytoplasm. The oocytes were

previously stained in H199 medium containing 10% FCS, 5 μg/ml Hoechst 33342 and 7.5 μg/ml cytochalasin B (Sigma) for 5-10 minutes and manipulated in this medium but without Hoechst 33342. Enucleation was confirmed by visualising the karyoplast under ultraviolet light. Following enucleation, the resulting cytoplasts were washed extensively in H199 + 10% FCS and held in this medium until injection of donor cells.

c) **Preparation of quiescent (G0) donor cells** Cultured follicular cells were induced to enter a period of quiescence by serum deprivation (Campbell *et al.*, 1996b). One day after routine passage, the culture medium was aspirated and the cells washed three times with fresh changes of PBS before fresh culture medium containing only 0.5% FCS was added. The follicular cells were returned to culture for a further 9-23 days (commonly 10 days) in low serum before they were used for nuclear transfer. Immediately before injection, a single cell suspension of the donor cells was prepared by standard trypsinization. The cells were pelleted and resuspended in H199 + 0.5% FCS and remained in this medium until injection.

d) **Preparation of G1 donor cells** Proliferating follicular cells were cultured in an appropriate medium (for example DMEM/F12 plus 10% fetal calf serum) on glass coverslips in the culture dish. These coverslips containing the cells growing on their surface may be physically picked out of the culture dish in a sterile manner and placed into a suitably constructed micro-manipulation chamber, to enable cell or nuclei collection and injection. A droplet of HEPES-buffered medium containing 10% FCS is placed onto the cells which is then overlaid with mineral oil. So long as the density of the cells on the coverslip is not too high, it is possible to identify and with care, physically pick the mitotic cells off the coverslip with a manipulation pipette since they have rounded up and are only loosely attached to the culture surface during mitosis. If this proves difficult, one may briefly wash the cells with PBS before introducing a dilute concentration of trypsin solution (such as at one-tenth the strength used for routine sub-cultivation of the cultured cell line) containing 1.5 μg/ml cytochalasin B, principally to minimise any mechanical damage induced during physical removal of the cells from the coverslip. With suitable microscopy (for example phase contrast or

DIC optics) mitotic cells are identified on the coverslip primarily by visualising condensed chromatin on a mitotic spindle or by identifying condensed chromatin in a cell doublet, still connected by a cytoplasmic bridge, undergoing telophase stage of mitosis. Thus, there is no need to use a DNA specific flurochrome such as Hoechst 33342 and expose cells to UV light. With the aid of the injection pipette mounted on the manipulator, these mitotic cells are individually picked off the coverslip and placed into an adjacent droplet of HEPES-buffered medium containing 10% FCS to enable complete mitosis and eventual cell cleavage to form a doublet of cells. The diameter of the pipette should be of a suitable size, dependent upon the cell line, so as to not physically damage the cell or the spindle during manipulation. Thus, mitotic cells preferably in anaphase or telophase stages are individually selected, removed and allowed to complete mitosis and cleave in two. These cell doublets are then gently separated into individual cells which may be easily achieved by brief exposure to a suitable enzymatic solution. Each intact cell is then injected and fused to the cytoplast. Alternatively the cell nucleus may be isolated and injected directly into the cytoplasm of the enucleated oocyte. Preferably the introduction of the donor nucleus into the cytoplast is completed within three hours of originally picking the mitotic cell off the culture surface. This ensures that the cultured cells are fused at an early G1 stage of the cell cycle and before S-phase occurs. With each individual cell type or cell line used this should be confirmed by, for example, negative BrdU labeling of a sample of the selected cells. In addition, confirmation that the cells so picked by this method are in fact cycling and do enter S-phase at some latter point is recommended.

e) **Microinjection** Recipient cytoplasts were dehydrated in H199 containing 10% FCS and 5% sucrose. This medium was also used as the micro-manipulation medium. A suitably-sized pipette (e.g. 30-35 μm external diameter), containing the donor cell, was introduced through the zona pellucida and the cell wedged between the zona and the cytoplast membrane to facilitate close membrane contact for subsequent fusion. Following injection, the reconstructed embryos were rehydrated in two steps; firstly in H199 containing 10% FCS and 2.5% sucrose for 5 minutes and then in H199 + 10% FCS until fusion.

f) **Cell fusion** For both the G0 and G1 cell treatments embryos were reconstructed using the FBA (fusion before activation) strategy. Reconstructed embryos were electrically fused at approximately 24 hours post-start of maturation (hpm) in buffer comprising 0.3 M mannitol, 0.5 mM HEPES and 0.05% fatty acid free (FAF) BSA with 0.05 mM calcium and 0.1 mM magnesium. Fusion was performed at room temperature, in a chamber with two stainless steel electrodes 500 μm apart overlaid with fusion buffer. The reconstructed embryos were manually aligned with a fine, mouth-controlled Pasteur pipette, so that the contact surface between the cytoplast and the donor cell was parallel to the electrodes. For the follicular cells, cell fusion was induced with two DC pulses of 2.25 to 2.50 kV/cm for 15 μs each, delivered by a BTX Electrocell Manipulator 200 (BTX, San Diego, CA) (optimal electrical parameters need to be identified for each cell line). Following the electrical stimulus, the reconstructed embryos were washed in H199 + 10% FCS. They were then checked for fusion by microscopic examination within 15-30 minutes.

The electrical fusion parameters above are not expected to cause significant rates of activation with young cytoplasts used at 24 hpm, since less than 1% of control oocytes (n=112) at the same age formed pronuclei after a similar electrical stimulus (Wells *et al.*, 1999). This is important for the FBA treatment, so that NEBD and PCC do occur, allowing exposure of the donor chromatin of the G1 and G0 nuclei to factors present within the oocyte cytoplasm to enable chromatin remodeling and nuclear reprogramming.

Alternatively, the nuclei from cells in G1 may be isolated and injected directly into the oocyte cytoplasm as known by a person skilled in the art.

g) **Activation** There are a variety of methods to effect artificial activation. One particular method involves the combination of ionomycin (Sigma) and 6-dimethylaminopurine (6-DMAP; Sigma) (Susko-Parrish *et al.*, 1994). Following fusion, embryos were activated preferably after the donor nuclei have been exposed to

the oocyte cytoplasm for a period of 3-6 hours. This preferred method has been termed "fusion before activation" (FBA; Wells *et al.*, 1998). Thirty minutes before activation, fused embryos in the FBA treatment were washed and held in HSOF (containing calcium) + 1 mg/ml FAF BSA. Activation was induced by incubation in 30-μl drops of 5 μM ionomycin (Sigma) in HSOF + 1mg/ml FAF BSA for 4 minutes at 37° C. Activation commonly occurred in cytoplasts aged between 27-30 hpm. Embryos were then extensively washed in HSOF + 30 mg/ml FAF BSA for 5 minutes before culture in 2 mM 6-dimethylaminopurine (6-DMAP; Sigma) for 4 hours in AgR SOF (plus calcium) + 10% FCS.

The improved rates of embryo development that result from a period of prolonged exposure of the nucleus to oocyte cytoplasm as in the FBA methodology (Stice *et al.*, 1996; Wells *et al.*, 1998; 1999) must be combined with suitable treatments to prevent micronuclei formation occurring following such delayed activation (Czolowska *et al.*, 1984). A serine-threonine kinase inhibitor such as 6-DMAP appears to be one suitable reagent. 6-DMAP therefore allows for the formation of a single intact nucleus following the initial activation stimulus thus, maintaining the correct ploidy in the reconstructed embryo.

In Vitro Culture of Nuclear Transfer Embryos

Embryo culture was performed in 20 μl drops of AgR SOF (commercially available from AgResearch, Hamilton, New Zealand) overlaid with paraffin oil. AgR SOF is a modified formulation of SOFaaBSA (containing 8 mg/ml FAF BSA; as described by Gardner *et al.*, 1994). Whenever possible, groups of up to 10 embryos were cultured together in droplets of medium. Embryos were cultured in a humidified modular incubator chamber (ICN Biomedicals, Aurora, OH) at 39° C in a 5% CO_2: 7% O_2: 88% N_2 gas mix. On Day 4-5 of development (Day 0 = day of embryo reconstruction), embryos were transferred to fresh 20 μl drops of AgR SOF plus 10 μM, 2, 4-dinitrophenol acting as an uncoupler of oxidative phosphorylation to improve the *in vitro* development of bovine embryos as taught in published patent specification No.

WO 00/38583, which is incorporated herein by reference. On Day 7 post fusion, development to transferable-quality blastocysts was assessed.

Embryo Transfer, Pregnancy Diagnoses and Calving

The transfer of embryos, diagnosis of pregnancy and calving management were carried out using skills well known in the art.

Serial Nuclear Transfer and Recloning

In a further embodiment of the invention, it may be desirable to reclone the first generation cloned embryos originally produced by reconstructing a donor cell in G1 with a suitable recipient cell. Recloning may be achieved by disaggregating a pre-implantation stage embryo into individual cells and then fusing each of these to suitable recipient cytoplasts. As would be appreciated by a person skilled in the art, this form of embryonic cell (or blastomere) cloning requires co-ordination of the cell cycles between the donor and recipient cells to avoid chromosomal abnormalities and optimise development. Preferably donor cells are obtained when the first generation cloned embryo is at the morula stage (approximately 32 cells in cattle) but the embryo could be at either earlier or later stages of development. Alternatively, one could firstly generate a cloned fetus produced from a G1 cultured donor cell, then re-derive a fetal cell line and reclone embryos using this new cell line. This recloning approach may provide an advantage in nuclear reprogramming by prolonging the period of exposure of the original donor nucleus to oocyte cytoplasm during the early pre-implantation period. It also has the advantage of multiplying the numbers of cloned embryos available from the first generation founder embryo, to produce cloned embryos in the second generation, third generation and so on.

Serial nuclear transfer involves the sequential transfer of nuclei to suitable recipient cell cytoplasmic environments. As an embodiment of the present invention, it may be desirable to firstly reconstruct a one-cell embryo with a G1 donor cell and a non-

activated recipient cytoplast high in MPF. This will allow NEBD and PCC and when followed by an activation stimulus, an intact diploid nucleus will be formed. This nucleus (that has undergone nuclear remodeling and a degree of nuclear reprogramming) may then be aspirated as a karyoplast from the one-cell cloned embryo and be sequentially transferred to an enucleated zygote at an appropriate stage post-fertilisation. Embryo development is then allowed to proceed. Such a sequential serial nuclear transfer process may improve nuclear reprogramming. It may also result in improved development as a result of the second nuclear transfer step introducing the nucleus into a cytoplasmic environment which has been more appropriately activated into embryonic development as a consequence of having undergone fertilisation with a sperm.

Production of Transgenic Animals

The production of transgenic livestock following nuclear transfer with genetically modified donor nuclei at the G1 stage of the cell cycle will likely be a more efficient and versatile approach that pronuclear injection of DNA into zygotes (Wall *et al.*, 1997) or sperm-mediated transgenesis involving intra-cytoplasmic injection of sperm and exogenously-bound DNA (Perry *et al.*, 1999). The advantages of the nuclear transfer approach with cultured cells include; (1) a far wider range of genetic manipulations are possible; (2) it more readily enables genetic manipulation on a high genetic background using a cell line as opposed to collecting oocytes or zygotes; (3) all of the resulting cloned offspring are transgenic and of the desired sex; and (4) there is the opportunity to produce instant flocks and herds generating useful product in a shorter timeframe compared to producing individual founder animals with the pronuclear injection approach or sperm-mediated transgenesis.

Cultured cells may be genetically altered by any known method to insert, remove or modify a desired DNA sequence. Modifications include the random insertion of new DNA sequences (which may be heterologous), site specific insertion of DNA and homologous recombination which allow the insertion, deletion or modification of a

DNA sequence at a specific site in the genome. Following cell selection and DNA analyses to verify the desired genetic modification using methods known in the art, karyotypically normal transgenic cells may then be selected in G1 phase of the cell cycle and used for nuclear transfer to generate cloned / transgenic animals.

There are a wide variety of opportunities available for genetically modifying livestock for both biomedicine and agriculture, depending upon the specific genes that are manipulated. The areas of opportunity include; (1) production of pharmaceutical proteins in milk, blood or urine; (2) production of nutraceutical products and medical foods, for example in milk; (3) manipulation of agricultural production traits, for example improving the quantity and quality of milk, meat and fibre and improving disease and pest resistance, (4) production of industrial proteins in milk, for example; (5) xenotransplantation; (6) generating livestock as models for human disease, for example cystic fibrosis and Huntington's disease.

Therapeutic Cloning

A significant impact of nuclear transfer and embryonic stem cell technologies may be in the area of human cell-based therapies (Pedersen, 1999). Patients with particular diseases or injuries in tissues which neither repair nor replace themselves effectively (as occurs in diabetes, muscular dystrophy, spinal cord injury, certain cancers and various neurological disorders, including Parkinson's disease, etc) could potentially generate their own therapeutic tissue for transplantation, offering prolonged or lifelong treatment. Initially, this approach would employ human nuclear transfer. This may involve collecting a small sample of healthy tissue from a human patient suffering a particular disease or injury and stimulating the proliferation of cells in culture. By selecting donor cells in G1 of the cell cycle and fusing them to a suitable recipient cell it may be possible to reprogramme the nucleus. If the recipient cell were an enucleated human oocyte then the reconstructed embryo, following a suitable activation stimulus, could be grown in an appropriate embryo culture medium to the blastocyst stage. Under suitable culture conditions (Thomson *et al.*, 1998) human embryonic stem cells

may then be derived from the inner cell mass of such a cloned embryo which would be genetically identical to the patient which donated the cultured cells.

By "embryonic stem cells" we mean cells isolated from any pluripotent cell types present within the embryo, and are preferably isolated from the inner cell mass of the blastocyst stage embryo by methods well known in the art.

The embryonic stem cells so produced by the method of the invention would be undifferentiated, pluripotent (potentially capable of differentiating into almost any cell type in the body) and possess essentially unlimited proliferative capacity *in vitro*. Alternatively, cell lines isolated from the inner cell mass may be somewhat more differentiated and possess a more limited ability to differentiate into a wide variety of cell types, but may still be therapeutically useful. Based on experience with mouse embryonic stem cells, suitable conditions can be developed which enable the production of purified populations of specific differentiated cell types such as nerve cells, haematopoietic cells, cardiomyocytes, etc to treat specific disorders (*eg* insulin-producing pancreatic cells for diabetes or dopamine-producing nerve cells for Parkinson's disease). These genetically compatible cells could then be administered back to the human patient, in order to regenerate normal tissue *in situ* following transplantation. Because the cells are genetically identical to the patient they will not be rejected and so there would be no or little need for immuno-suppressive drugs. It may also prove beneficial to generate "universal" embryonic stem cell lines for allogenic transplantation following systematic modification of loci such as major histocompatability complex genes that play an important role in the recognition of foreign cells by the immune system.

Some applications may involve the genetic modification of the embryonic stem cells prior to differentiation and transplantation. This may be for the purposes of gene therapy to deliver a therapeutic drug for treatment or to correct a genetic defect in somatic cells such as occurs in the dystrophin gene in the skeletal muscle of patients with Duchenne muscular dystrophy.

The differentiation of human embryonic stem cells to specific cell types may also be beneficial for drug discovery and toxicology studies for human medicine, in addition to transplantation therapy with cells, tissue or organs.

In addition, cells, tissues and organs may be isolated from cloned non-human animal offspring, and used for transplantation in human patients in need of such therapy (xenotransplantation). Where such cells, tissues or organs comprise a transgene, such cells, tissues or organs may be useful in gene therapy or to moderate the patient's immune response to the xenogenic tissue.

Recipient cells for human applications

In the method of nuclear transfer according to the invention, the preferred recipient is an enucleated oocyte prepared by the method disclosed above. However, for human applications this may prove difficult.

An alternative source of recipient cells to reprogramme differentiated somatic nuclei may be embryonic stem cells. Thus, somatic cells from a patient requiring some form of cell-based therapy may be de-differentiated in culture without the requirement of human oocytes. This may be achieved by fusing a healthy somatic cell in G1 of the cell cycle to an enucleated embryonic stem cell or a group of embryonic stem cells (to provide a larger mass of cytoplasm for reprogramming). It would be necessary to control the state of the cell cycle of the recipient stem cells, preferably this would be at M-phase or G1-phase at the time of fusion. In this way, the differentiated nucleus of the somatic cell may be de-differentiated following exposure to the cytoplasm of the stem cell. The resulting reconstructed cell may have multipotent or pluripotent developmental potential, and may be induced to form an array of other specialised cell types useful for therapy. This concept has been previously demonstrated in hybrid cells produced by fusing thymic lymphocytes and embryonic germ cells (Tada *et al.*, 1997).

The invention will now be exemplified by the following examples which are not intended to limit the scope of the invention as would be appreciated by a person skilled in the art.

<u>Example 1.</u> Effect of follicular donor cells synchronised in either G0 or G1 on *in vitro* development following nuclear transfer.

In this particular experiment, a primary cell line of follicular granulosa cells were used for nuclear transfer. The cell line was denoted as "J1" and was derived from a New Zealand Jersey heifer. J1 cells were used in this experiment between passage numbers 7-8 of culture.

Donor cells were synchronised at two stages of the cell cycle for comparison:

(1) **G0 cells** were obtained following serum deprivation by culture in medium containing 0.5% FCS for 10-11 days.

(2) **G1 cells** were fused to cytoplasts within 1-3 hours following completion of mitosis.

Negative BrdU labeling confirmed that J1 cells fused within 3 hours of mitosis had not entered S-phase and were in G1 of the cell cycle.

Embryos reconstructed with donor cells from the two cell cycle treatment groups in this experiment were cultured *in vitro* in AgR SOF medium supplemented with Life Technologies BSA (Life Technologies product number 30036-578).

Results of *in vitro* development are presented in Table1. Donor cells in G0 (control) and G1 stage of the cell cycle were equally efficient at fusing with the cytoplast (recipient cell) by means of electrically-mediated fusion. There was also no difference in the proportion of fused reconstructed embryos placed into *in vitro* culture that

developed to blastocysts between the G0 and G1 donor cell treatment groups as shown in Table 1, below.

Table 1: *In vitro* development of cloned embryos reconstructed with J1 follicular cells at either G0 or G1 stages of the cell cycle and cultured in AgR SOF medium supplemented with Life Technologies BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=152)**	**86 ± 5.2%**	**24 ± 1.1%**	**77 ± 6.4%**
G1	**(n=186)**	**78 ± 5.0%**	**25 ± 6.2%**	**77 ± 6.9%**

Example 2. Effect of follicular donor cells (J1 and EFC cell lines) synchronised in either G0 or G1 on *in vitro* development following nuclear transfer and cultured in medium supplemented with Sigma BSA.

Further evidence is provided in Table 2 to support the finding that there is no effect of stage of cell cycle between G0 and G1 follicular donor cells on *in vitro* development of cloned embryos to the blastocyst stage following 7 days culture.

Experiments were performed with two independent follicular cell lines:

(1) "EFC"; a follicular cell line from a New Zealand Friesian dairy cow and
(2) "J1" a follicular cell line from a New Zealand Jersey dairy heifer.

EFC cells were only used in G0 while J1 cells were only used in G1 of the cell cycle in these experiments. However, the data has been combined in this example since both cell lines are derived from follicular cells and the reconstructed embryos were all cultured in the same media formulation. In these experiments, this was the standard

AgR SOF medium, but supplemented with Sigma BSA (Sigma Chemical Company; product number A-7030) rather than Life Technologies BSA, as in example 1 above.

J1 cells were used for nuclear transfer between passages 3-6, with donor cells fused within 1-3 hours following the completion of mitosis. EFC cells were used between passages 3-8 of culture and were synchronised in a G0 stage by culture in medium containing 0.5% serum for between 9-18 days.

The *in vitro* development results, presented in Table 2, show that with follicular donor cells there is no difference in the proportion of fused embryos developing to blastocysts between G0 and G1 cell cycle stages.

Table 2. *In vitro* development of cloned embryos reconstructed with follicular cells (J1 or EFC) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with Sigma BSA (mean ± s.e.m.).

Cell line	*Stage of cell cycle*		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
J1	**G1**	**(n=576)**	**82 ± 3.8%**	**38 ± 3.6%**	**67 ± 1.8%**
EFC	**G0**	**(n=1108)**	**78 ± 2.3%**	**37 ± 2.3%**	**59 ± 3.3%**

Example 3. Effect of follicular donor cells (J1 and EFC cell lines) synchronised in either G0 or G1 on *in vivo* development following nuclear transfer.

Data is presented in Figure 1 that shows the survival throughout gestation, following the transfer to recipient cows, of cloned bovine embryos reconstructed with follicular cells either in G0 or G1 of the cell cycle. The data in Figure 1 has been complied from embryos that were produced from experiments illustrated in examples 1 and 2 above. This includes cloned embryos generated from two follicular cell lines, namely J1 and

EFC. Additionally, it includes embryos that have been produced in the same AgR SOF media formulation but supplemented with either Sigma BSA or Life Technologies BSA. The data have been pooled since there is no effect on post-transfer viability between either of these two follicular cell lines nor an effect of the two sources of BSA (although there is a significant effect on development to the blastocyst stage).

The data in Figure 1 represents a total of 85 embryos reconstructed with G0 donor cells and 95 embryos reconstructed with early G1 donor cells that were transferred to the reproductive tracts of synchronised recipient cows.

Figure 1 plots the percentage of embryos / fetuses present throughout gestation, from day 7 embryo transfer to term, as determined by regular ultrasonography, rectal palpation and calving. Most notably, the use of G1 follicular cells resulted in the birth of viable calves at full term. Compared to G0 donor cells, there was a tendency for embryonic survival with cloned embryos reconstructed with G1 cells to be lower from day 30 of gestation right through to full term, however, this did not reach a level of statistical significance with the numbers of transfers reported here.

With G1 follicular cells, 9% of embryos transferred (9/95) resulted in the birth of calves at full term. However, 4 of these calves died at or shortly after birth resulting in an overall efficiency of 5% viable cloned calves from G1 cells (5/95). In comparison, G0 donor follicular cells resulted in 20% development to full term (17/85), with 3 calves dying during birth culminating in an overall 16% efficiency of viable calves produced (14/85).

<u>Example 4.</u> Effect of adult female skin fibroblasts (Age + and Age - cell lines) synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer and culture in medium supplemented with Life Technologies BSA.

Data have been combined from experiments with two independent adult skin fibroblast primary cell lines. The two cell lines are denoted "Age +" and "Age –". They are both female cell lines from Angus beef cows selected for either late or early onset to puberty, respectively. As there were no significance differences in terms of the *in vitro* and *in vivo* development between these two similar cell lines, the data have been combined in Table 3 and Figure 2 below.

Two stages of the cell cycle were compared in these experiments:

(1) **G1**, whereby donor cells were fused to cytoplasts within 1-3 hours post completion of mitosis.

(2) **G0**, whereby donor cells where cultured for 3-12 days in medium supplemented with 0.5% FCS. For those reconstructed embryos that were transferred to recipient cows, cells were deprived of serum for 4-5 days.

Cells from both cell lines and both cell cycle treatments were used for nuclear transfer at passage 7 of culture. Reconstructed embryos where cultured in the standard AgR SOF media formulation, supplemented with Life Technologies BSA (Life Technologies product number 30036-578).

The data presented in Table 3 show an effect of length of time G0 cells were in low serum on fusion efficiency, however, once fused with the cytoplast there was no effect on subsequent *in vitro* development and so, the G0 blastocyst data have been combined. There was no effect of cell cycle stage between G0 and G1 on development to blastocyst stages.

Table 3. *In vitro* development of cloned bovine embryos reconstructed with adult female skin fibroblast cells (Age + and Age -) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with Life Technologies BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**3-5 days (n=411)**	**66 ± 1.9%[a]**	**19 ± 3.0%**	**58 ± 4.5%**
	7-12 days (n=107)	**41 ± 6.9%[b]**		
G1	**(n=401)**	**59 ± 2.5%[ab]**	**19 ± 5.0%**	**67 ± 6.6%**

ab $P<0.05$

The data in Figure 2 demonstrate that adult skin fibroblasts selected in G1 are capable of producing viable calves at full term. Similar to the data in Figure 1, there is a tendency for survival at full term to be greater with G0, however, from the numbers of transfers performed here this is not statistically significant. With G1 donor cells, 4% of embryos transferred yielded viable cloned calves at term (1/25). In comparison, G0 donor cells resulted in 14% viable development to term (3/22). In this experiment, all 4 calves delivered survived the post-natal period.

Example 5. Effect of adult skin fibroblasts (LJ801 and 3XTC cell lines) synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer and culture in medium supplemented with ICP BSA.

Additional experiments comparing G0 and G1 cell cycle stages with two independent adult skin fibroblast cell lines were conducted. The two cell lines were denoted "LJ801" and "3XTC". LJ801 is a male cell line derived from a Limousine X Jersey

steer, while 3XTC is derived from a crossbred cow which on three occasions has delivered triplet calves.

Two cell cycle stages were compared:

(1) **G0 cells**, whereby donor cells in both cell lines were cultured in medium with 0.5% FCS for 4-5 days; and

(2) **G1 cells**, whereby donor cells were fused to cytoplasts within 1-3 hours following the completion of mitosis.

Negative BrdU labeling confirmed that adult skin fibroblast cells fused within 3 hours of mitosis had not entered S-phase.

Both LJ801 and 3XTC were used for nuclear transfer experiments between passages 3-4 of culture.

The data in Table 4 have been combined since there was no difference in the efficiency of development to blastocyst stages between LJ801 and 3XTC cells and reconstructed embryos from both cell lines were cultured in the same standard AgR SOF media but supplemented with an alternative BSA source, this time from ICP (Immuno-Chemical Products, Auckland, New Zealand; product number ABFF-002). The data on embryonic survival throughout gestation, following embryo transfer, is presented in Figures 3 and 4 in terms of the respective cell lines 3XTC and LJ801.

The data in Table 4 show that there was no effect of the cell cycle of donor adult skin fibroblasts synchronised in either G0 or G1 on development to the blastocyst stage.

Table 4. *In vitro* development of cloned embryos reconstructed with adult skin fibroblast cells (LJ801 and 3XTC cells) at either G0 or G1 of the cell cycle and cultured in AgR SOF medium supplemented with ICP BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=145)**	**72 ± 7.8%**	**36 ± 6.1%**	**61 ± 5.0%**
G1	**(n=200)**	**60 ± 3.1%**	**37 ± 6.0%**	**57 ± 5.0%**

The data in Figure 3 show that with embryos reconstructed with 3XTC cells that there was no difference in survival to day 150, at least. With donor cells in G1 of the cell cycle embryonic survival at day 150 was 25% (3/12) compared to 27% for G0 donor cells (3/11).

The data in Figure 4 show that with embryos reconstructed with LJ801 fibroblast cells that whilst there is a trend for embryonic survival to be greater at day 210 of gestation with G0 donor cells, this is not statistically significant. With donor cells in G1 of the cell cycle embryonic survival at day 210 was 23% (3/13) compared to 39% for G0 donor cells (7/18).

Example 6. Effect of genetically modified bovine fetal fibroblast cells synchronised in either G0 or G1 on *in vitro* and *in vivo* development following nuclear transfer.

An experiment was also performed with genetically modified bovine fetal female lung fibroblast cells to investigate the effect of cell cycle stage on development following nuclear transfer. The genetic modification involved the random insertion of additional

copies of bovine β and κ-casein genes. The transgenic cell line was denoted "casein plus 5110".

Two cell cycle stages were compared:

(1) **G0 cells**, whereby donor cells were cultured in medium with 0.5% FCS for 3-6 days; and

(2) **G1 cells**, whereby donor cells were fused to cytoplasts within 1-3 hours following the completion of mitosis.

Reconstructed embryos were cultured in the standard AgR SOF media formulation, supplemented with ICP BSA (Immuno-Chemical Products, Auckland, New Zealand; product number ABFF-002).

The data on *in vitro* embryo development is presented in Table 5. Development to blastocyst stages with the casein plus 5110 cells was significantly less when donor cells used for nuclear transfer were in G1 compared to G0. This result is in contrast to the other cell lines presented in the previous examples above.

Table 5. *In vitro* development of cloned cattle embryos reconstructed with transgenic female fetal lung fibroblast cells (casein plus 5110 cells) at either G0 or G1 stages of the cell cycle and cultured in AgR SOF medium supplemented with ICP BSA (mean ± s.e.m.).

Stage of cell cycle		*Fusion*	*Grade 1-2 Blastocysts*	*Total Development*
G0	**(n=150)**	**90 ± 8.1%**	**52 ± 1.8%[a]**	**72 ± 2.9%[c]**
G1	**(n=73)**	**77 ± 6.5%**	**26 ± 5.1%[b]**	**43 ± 2.2%[d]**

ab $P<0.05$; cd $P<0.01$

The embryonic survival data presented in Figure 5 shows no effect of stage of cell cycle or development to day 90 at least, with casein plus 5110 transgenic cells. With donor cells in G1 of the cell cycle embryonic survival at day 90 was 38% (9/24) compared to 27% for G0 donor cells (6/22).

Example 7. Effect of non-proliferating, senescent donor cells (in late G1 phase) on in vitro and in vivo development following nuclear transfer.

A nuclear transfer experiment was performed to investigate the effect on development with non-proliferating, senescent bovine donor cells. The cells used in this experiment were fetal female lung fibroblasts and had been genetically modified (denoted as "561 cells"). Initially, the cells were actively growing, however, during the course of their culture this progressively slowed and at late passage the cells entered a non-proliferative phase, known by those skilled in the art as senescence, whereby cell division ceases. Senescent cells are known to arrest in G1 of the cell cycle, specifically at the late G1 / S-phase boundary (Sherwood et al., 1988). When cells enter senescence they block at late G1 and they fail to enter S-phase in response to physiological mitogens. Thus, senescence is distinct from quiescence, whereby in the latter situation cells may be induced to re-enter the cell cycle and proliferate upon return to optimal conditions (eg addition of serum in the case of serum deprived cell cultures).

Embryos reconstructed with transgenic senescent cells were cultured in the AgR SOF media, supplemented with ICP BSA.

The data on in vitro embryo development is presented in Table 6. Development to blastocyst stages with the senescent donor cells was lower than that expected for G0 or early G1 donor cells as illustrated in the previous examples.

Table 5. In vitro development of cloned cattle embryos reconstructed with non-proliferating, senescent transgenic fibroblasts (561 cells) and cultured in AgR SOF medium supplemented with ICP BSA.

Cell line	Stage of cell cycle	Fusion	Grade 1-2 Blastocysts	Total Development
561	**Senescent (n=158)**	**88%**	**12%**	**35%**

The embryonic survival data presented in Figure 6 suggests a low efficiency of cloning with non-proliferating, senescent cells arrested in late G1 phase, with only 4% of embryos developing to day 90 (1/26).

CONCLUSIONS

1. It is possible to select individual cells at a definitive stage of the cell cycle, preferably early G1. This is advantageous compared to prior art using so-called proliferating cells where the actual stages of the cell cycle are not accurately known for each individual cell used for nuclear transfer. The same is true for serum starved populations of cells.

2. Post-mitotic cells in the early G1 phase of the cell cycle are totipotent following nuclear transfer as evidenced by the production of viable calves.

3. Thus, G0 is not the only stage of the cell cycle that is compatible with development following nuclear transfer with differentiated cultured cells and G1, preferably early G1, nuclei can also be functionally reprogrammed.

4. Post-mitotic early G1 cells promote development to the blastocyst stage following nuclear transfer to similar levels as cells in G0.

5. There is no significant difference in the post-transfer viability to full term of cloned embryos reconstructed with either G0 or early G1 donor nuclei.

INDUSTRIAL APPLICATION

The present invention may be useful in establishing cloned herds/flocks of animals including transgenic animals capable of producing pharmaceutically useful proteins, agriculturally useful products such as meat, milk and fibre, as well as human applications especially in the field of therapeutic cloning.

It will be appreciated that the description is not intended to limit the scope of the invention to the above examples only, many variations such as might readily occur to a person skilled in the art being possible, without departing from the scope of the appended claims.

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All references are incorporated herein in their entirety by reference.